Remarks

Applicants note with appreciation the Examiner's continued acknowledgement of the allowance of Claims 1-10 and 12-15.

Applicants further note with appreciation the acknowledgement of the claim for foreign priority based on French Patent Application No. 94/01015, filed January 31, 1994 (Publication No. 2 715 664). On September 29, 2003, Applicants submitted a claim for priority under 35 U.S.C. §119, enclosing a certified copy of French Patent Application No. 94/01015, filed January 31, 1994. In accordance with the Examiner's helpful comments, the Applicants submit herewith a certified copy of the English translation of French Patent Application No. 94/01015.

Claim 11 has been rejected as anticipated by both Carayannopoulos et al. (PNAS, August 1994, Vol. 91, pp. 8348-8352). Further, Claim 11 has been rejected under the doctrine of double patenting over claims 14-17 of U.S. Patent No. 6,312,690.

Applicants respectfully submit that as a result of the claim for foreign priority based on French Application 94/01015, both Carayannopoulos et al. and U.S. Patent 6,312,690 are not applicable as prior art. Neither publication predates the Applicants' French priority date of January 31, 1994. The publication date of Carayannopoulos is August 1994. The earliest possible date for U.S. Patent 6,312,690 is September 1, 1995. The Applicants' French priority Application 94/01015 provides the Applicants' with a priority date of January 31, 1994. Support for the subject matter of Claim 11 can be found on page 5, lines 14-15 of the Certified copy of the English translation of French Patent Application No. 94/01015.

In view of the foregoing, neither publication qualifies as prior art and the Applicants respectfully request withdrawal of the anticipation rejection of Claim 11 under Carayannopoulos et al. and the double patenting rejection of Claim 11 over U.S. Patent 6,312,690.

In view of the foregoing, Applicants respectfully submit that the Application is now in condition for allowance, which is respectfully requested.

Respectfully submitted,

T. Daniel Christenbury
Reg. No. 31,750
Attorney for Applicants

TDC/JEB:pam
(215) 656-3300

Abbaye Traductions
www.abbaye.com
37, Rue Amsterdam – 75008 PARIS – Tél : 33 (1) 44.53.99.39 – Fax : 33 (1) 49.95.94.78

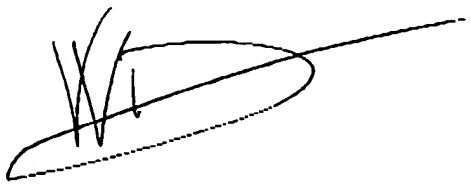
VERIFICATION OF TRANSLATION

I, Mrs WOOD (name of translator)

Of ***Abbaye Traductions 37, rue Amsterdam 75008 PARIS*** *(translator's address) hereby declares that I am conversant with the French and English languages and that I am the translator of the* ***French Patent Application N° 9401015 of January 31, 1994 (Publication N° 2 715 664)****, and I certify that to the best of my knowledge and belief these are true and correct English translations.*

Dated October 6, 2003

Signature of translator



RECOMBINANT BACULOVIRUS AND ITS USE FOR THE PRODUCTION OF MONOCLONAL ANTIBODIES

The present invention relates to a modified baculovirus and to its use for the production of immunoglobulins.

Antibodies or immunoglobulins are produced by B lymphocytes. Each B lymphocyte secretes a single type of antibody. Each immunoglobulin molecule is constituted of the combination of two heavy chains (H) and two light chains (L) connected by disulfide bridges. Each chain is constituted of a variable region (VH and VL) which contains the antigen attachment site and a constant region (CH and CL). There are many types of heavy chains (γ1, γ2, γ3, γ4, α, ε, μ) which define the various classes of immunoglobulins (IgG1, IgG2, IgG3, IgG4, IgA, IgE, IgM ...) and two types of light chains: kappa chain (κ) and lambda chain (λ). For example, the IgG1 class antibodies are constituted of two heavy chains of the γ1 type and two kappa κ or lambda λ light chains. The variable regions are the support of the specificity of the antibody for its antigen.

For each chain of a given antibody, the variable region is constituted of many domains, certain of which are preserved to varying degrees. The rearrangement of these variable regions is the fruit of a recombination at the level of the genomic DNA of the B lymphocytes.

Monoclonal antibodies are conventionally produced from cultures of hybridoma lines, with each line derived from a single B lymphocyte and secreting a single type of immunoglobulin.

Monoclonal antibodies (MAbs) are commonly used at present for in vitro diagnostics, and their use in therapy and for in vivo diagnostics shows promising developments. These developments, however, are held back by the fact that the only monoclonal antibodies that ar relatively easily available in adequate quantities from hybridoma cultures are monoclonal antibodies from

rodents. However, these rodent immunoglobulins (and nonhuman immunoglobulins in gen ral) induce an und sirable immune response in humans which considerably limits their therapeutic value.

Extensive research has been carried out with the goal of obtaining immunoglobulins that do not have this drawback; specifically, it has been proposed to employ genetic engineering techniques to fabricate recombinant antibodies in which the largest possible part of the molecule is derived from a gene of human origin.

The resultant antibodies, in which only the variable domains are of nonhuman origin, are referred to as chimeric antibodies. There also exist antibodies referred to as human in which the sequences of th.e variable regions not directly involved in the recognition of the antigen have been replaced by sequences of human origin. In both cases, the greatest part of the immunoglobulin molecule is derived from a gene of human origin.

Nevertheless, the production of antibodies using genetic engineering still requires the selection of a suitable host of expression in order to assure the post-translational modifications required to reproduce the properties of the native antibody. For this purpose, it has been proposed, among other approaches, to employ the baculovirus/insect cell system.

Baculoviruses are widely used as vectors for the expression of heterologous genes, placed under the control of viral promoters, in the cells of infected insects. The promoter of polyhedrin or of pIO (proteins produced in large amounts during the late phase of the viral replication cycle) is thus frequently used for this purpose.

HASEMAN and CAPRA [Proc. Natl. Acad. Sci. USA, 87,3942-3946 (1990)], PUTLITZ et al. [Bio/Technology, 8, 651-654 (1990)], REIS et al. [Bio/Technology, 10,910-912 (1992)] thus constructed baculoviruses in comprising two copies of the polyhedrin promoter, with one of these copies controlling the expression of a gene coding for the heavy chain of a mouse

immunoglobulin and the other copy controlling the expression of a gene coding for the light chain of the same immunoglobulin. The insect cells infected by these baculoviruses secreted immunoglobulins that had essentially the same properties as the antibodies of lymphocytic origin employed as the model. However, the structure of the baculoviruses modified in this manner is not maintained beyond several cycles of viral replication.

The goal of the present invention is to produce both the heavy chain (H) and the light chain (L) of a given antibody in an insect cell, by employing an expression vector derived from a baculovirus that does not exhibit the drawbacks of the expression vectors of the same type used in the prior art for the production of immunoglobulins.

To attain this goal, the inventors constructed double-recombinant baculovirus in which the coding sequence of each of the two H and L chains is placed under the control of a different strong promoter.

The object of the present invention is a recombinant baculovirus, characterized in that it comprises:

- an expression cassette comprising a sequence coding for at least one part of an immunoglobulin H chain, which sequence is placed under transcriptional control of a first strong baculovirus promoter or a derivative of said first promoter and

- an expression cassette comprising a sequence coding for at least one part of an immunoglobulin L chain, which sequence is placed under transcriptional control of a second strong baculovirus promoter or a derivative of said second promoter;

with the first and second promoters being two different promoters or derivatives of different promoters, one of the promoters is situated at the site occupied in the wild baculovirus by the polyhedrin promoter and the other is situated at the site occupied in th wild baculovirus by the p10 promoter.

Examples of strong promoters which can be used in the scop of the present invention are the promoters of polyhedrin and PI0 of the AcMNPV or SIMNPV baculoviruses. Moreov r, « d rivative of promoter » must be understood as synthetic or recombinant promoter obtained from a baculovirus promoter and functional in insect cells; such as, for example, the synthetic promoter described by WANG et al. [Gene, 100, 131-137 (1991)].

According to a first mode of realization of the present invention, each expression cassette comprises: (i) a strong baculovirus promoter as deflned above; (ii) a sequence coding for a signal peptide; (iii) a sequence coding for a variable domain of an H or L immunoglobulin chain; (iv) a sequence coding for at least one part of a constant domain of an H or L immunoglobulin chain.

Such a recombinant baculovirus constitutes an expression vector that can be used directly for the production of immunoglobulins in an insect cell.

Preferably, the sequence coding for the signal peptide that is placed under the control of the first promoter and the sequence coding for the signal peptide that is placed under the control of the second promoter are two different sequences.

Numerous sequences that code for functional signal peptides in insect cells can be used for the implementation of the present invention. As nonlimitative examples, one can cite the sequences coding for the signal peptides of Drosophila acetylcholinesterase, of ovine trophoblastin, of bovine lactotransferrin, of the H and L chains of immunoglobulins, etc.

According to another mode of realization of a recombinant baculovirus according to the present invention, each expression cassette comprises: (i) a strong baculovirus promoter as defined above; (ii) a sequence coding for a signal peptide as defined above; (iii) at least one site allowing the insertion of a sequence coding for a variable domain of immunoglobulin between the sequence coding for the constant part and th sequenc coding for the signal

peptide; (iv) a sequence coding for at least one part of a constant domain of immunoglobulin.

Each site allowing insertion of the sequence coding for the variable domain is choosen in order to be single in the genome of the recombinant baculovirus. Such as, for example, the Bsu36I site can be inserted between the signal peptide sequence and the sequence coding for the constant part of heavy chain, and the Sse83-87I site between the signal peptide sequence and the sequence coding for the constant part of light chain.

Thanks to the insertion of a sequence coding for a variable domain of immunoglobulin in each of said sites, a expression vector according to the first mode of realization described above is obtained.

The sequences coding for the constant and variable domains can be of the same or different origins; it is also possible to use synthetic or recombinant sequences. Advantageously, the sequence coding for the constant domain is of human origin; the sequence coding for the variable domain can be of totally human origin or of at least partially nonhuman origin, for example, of murine origin, etc.

According to another preferred mode of realization of the present invention, one of the promoters is P10 promoter or one of its derivatives, and the other is the polyhedrin promoter or one of its derivatives.

The present invention encompasses insect cells infected with a recombinant baculovirus in accordance with the invention.

The infection of cells by a double-recombinant baculovirus in accordance with the invention results in the production of H and L chains. These chains join together to reconstitute the desired monoclonal antibody which is then secreted in the culture medium.

The present invention also has as its object a procedure for the preparation of an immunoglobulin, characterized in that one cultures the insect cells infected with a recombinant baculovirus in accordance with the

invention, and that one extracts the said immunoglobulin from the culture medium.

The pr sent invention also encompasses the immunoglobulins that can be produced by means of the aforementioned procedure.

The present invention also has as its object a procedure for the preparation of a recombinant baculovirus, which procedure is characterized in that:

- a first transfer plasmid comprising a sequence coding for at least one part of the immunoglobulin H chain, under transcriptional control of a first strong baculovirus promoter or a derivative of said first promoter is prepared;

- a second transfer plasmid comprising a sequence coding for at least one part of the immunoglobulin L chain, under transcriptional control of a second strong baculovirus promoter or a derivative of said second promoter is prepared;

with the first and second promoters being two different promoters or derivatives of different promoters;

and the homologous recombination of the two plasmids with the DNA of a baculovirus is implemented.

The construction of a recombinant baculovirus in accordance with the invention is implemented by using conventional heterologous genetic cloning techniques in baculoviruses.

Schematically, the construction of the transfer plasmids is implemented by insertion in a plasmid capable of replicating itself in a bacterial host (generally, E. coli) of the region of the baculovirus (for example, pl0 or polyhedrin) in place of which it is desired to insert the genes coding for the immunoglobulin H or L chains. In this region, the coding sequence of the baculovirus gene (and possibly the promoter sequence of the said gene) is r plac d by the s quence coding for the immunoglobulin chain to be expressed (and possibly by the promoter sequenc under th control of

which it is desired to express this immunoglobulin chain in the case, for exampl , of a "derivative" promot r). The transfer plasmid obtained in this manner thus contains an insert comprising a heterologous sequence flanked by baculovirus sequences. One then co-transfects the insect cells with the DNA of the resultant transfer vector and the DNA of the baculovirus, which by homologous recombination between the viral DNA and the baculovirus sequences flanking the heterologous sequence in the plasmid enables the transfer of the foreign sequence from the plasmid into the viral genome.

After replication of the viral DNA in the transfected cells, one next carries out selection of the recombinant baculoviruses that integrated the heterologous sequences.

According to a preferred mode of implementation of the procedure in accordance with the present invention, the transfer plasmids used carry an insert comprising: an expression cassette as defined above, and on both sides of this cassette, sequences of baculoviruses which are homologous with those of the regions flanking the portion of the viral genome by replacement of which one desires to insert the said cassette.

In accordance with a particularly avantageous feature of this mode of implementation, the said baculovirus sequences are homologous with those of the regions flanking the pl0 gene, or homologous with those of the regions flanking the polyhedrin gene.

Increased comprehension of the present invention will be provided on the basis of the description below which refers to examples of the preparation of recombinant baculovirus in accordance with the invention and to their use for the production of immunoglobulins in insect cells.

It should be understood, however, that these examples are presented solely for the purpose of illustrating the object of the invention and do not in any manner constitute a limitation.

Exampl 1: Con tru ti n of a Kappa light chain cas ette (pBCK) (Figur 1):

a- Plasmid pGmAc116T:

This transfer vector is derived from the plasmid pGmAc115T [ROVER et al., J. Virol., 66, 3230-3235 (1992)], which in turn is derived from the plasmid pAc1 [CHAABIHI et al., J. Virol., 67, 2664-2671 (1993)] containing the fragment EcoRI-I from the Autographa californica nuclear polyhedrosis baculovirus (AcMNPV) and thus the polyhedrin gene and the sequences flanking the said gene. In order to obtain pGmAc116T, the plasmid pGmAc115T was subjected to deletion of a 1900pb fragment extending from a site EcoRI located upstream of the polyhedrin gene to a site XhoI located 190Opb downstream of this site EcoRI. The deletion was implemented by exhaustive XhoI cleavage, followed by a partial cleavage by EcoRI. 5 µg of the plasmid pGM115T were digested for 2 hours at 37°C by 15 units of XhoI enzyme (Boehringer) in a reaction volume of 50 µl and under the conditions specified by the supplier. The enzyme was eliminated by an extraction with phenol/chloroform and the plasmid DNA was precipitated with alcohol. This DNA was then partially cleaved by EcoRI (Boehringer) in a reaction volume of 50 µl in the presence of 0.5 unit of enzyme. Incubation was carried out at 37°C for 20 minutes. After a new extraction with phenol/ chloroform, the extremities generated by the XhoI and EcoRI cleavages were made complete by Klenow's enzyme (Biolabs) in the presence of the 4 dNTPs in accordance with the protocol specified by the supplier. The plasmid DNA was then precipitated with alcohol and incubated with the ligase of the phage T4 (Boehringer) under the specified conditions. Competent E. coli bacteria were transformed by a part of the ligation mixture. Screening of the colonies from this transformation enabled selection of the plasmid pGmAcII6T.

b - The promoters:

The pIO promoter or the polyhedrin promoter from the Spodoptera littoralis nuclear polyhedrosis virus (SLMNPV) are amplified by PCR using primers enabling r constitution of a site EcoRV upstream of the promoter, and a site BglII downstream. The amplification product is digested by EcoRV and BglII, and the fragments carrying the promoter sequences are inserted in pGmAcII6T previously digested by the same enzymes. The digestion EcoRV and BglII makes it possible to eliminate the AcMNPV polyhedrin promoter and to replace it with one of the two promoters cited above.

The resultant plasmids are referred to respectively as pGmAcIO (pIO promoter) or pGmAc33 (polyhedrin promoter).

c - Signal peptide:

The coding sequence selected for the signal peptide is the following:

5' - ATG AAA CAC CTG TGG TTC TTC CTC CTC CTG GTG GCC GCT CCC AGA TGG GTC CTG TCG - 3'

This sequence was selected from the sequences published by Van ES, J.H., HEUTINK M., AANSTOOT, H., and LOGTENBERG T. [Journal of Immunology, Vol. 149, p. 492-497 (1992)].

This sequence was synthesized chemically in the form of two complementary oligonucleotides (OPP-HuPS-5' and OPP-HuPS-3') with ends allowing insertion of the duplex in a site BglII. At one of the ends of the duplex, there is a sequence corresponding to that of the beginning of the framework 1 of the light chains (with the site SacI) followed by a sequence carrying a site XhoI. Two modifications (underlined in the sequence above) were realized in order to optimize the codon use of said sequence in the baculovirus system. For the pairing, 15 µg of each of these two oligonucleotides are incubated in 50 µl of buffer (Tris 1 mM pH 7.5, EDTA 0.1 mM) for 5 minutes in a water bath at 70°C. The bath is then allowed to cool down to room temperature (22 to 25°C). Th mixture is used directly in the

ligation reactions with the plasmids pGmAc10 or pGmAc33 that hav previously be n cleaved by BglII.

The ligation conditions are as follows: 1 µg of the selected plasmid pGmAc, cleaved by BglII, 1 µg of the bicatenary oligonucleotide carrying the sequence coding for the signal peptide, 2 µl of 10X ligase buffer (BOEHRINGER), distilled water q.s.p. 19 µl, 1 unit (1 µl) of ligase (BOEHRINGER); incubation is carried out at 22°C for 2 hours; the ligation product is used for the transformation of competent E. coli bacteria.

d - Constant region :

The coding sequence of the constant region of the human κ light chain was amplified by PCR using as matrix human B lymphocyte cDNA. The human lymphocytes (circa 5 x 10^8) were prepared from 200 ml of blood using HISTOPAQUE® (SIGMA). The total RNA was extracted from these lymphocytes using a PHARMACIA kit (RNA Extraction Kit). The first cDNA strand was prepared from the total RNA using the "First-Strand cDNA Synthesis Kit" from PHARMACIA.

The primers used for amplification of the Cκ cDNA are as follows:

* HuCκBAC:

5'-AG CTC GAG ATC AAA CGG-3'

(the site XhoI is underlined).

This primer corresponds to a consensus sequence on 3' of the sequences coding for the variable domains of the light chains of murine immunoglobulins (Jκ) and contain an XhoI cleavage site.

* HuCκFOR:

5' - GAA GAT CTA ACA CTC TCC GCG GTT GAA G-3'

(the site BglII is underlined).

This primer is complementary with the end 3' of the human Cκ genes and carri s a sit BglII downstream of th stop codon TAG.

Amplification with the primers HuCκBAC and HuCκFOR produced a circa 340 pb fragment containing th totality of the Cκ r gion flanked by the sit s XhoI and BglII.

The amplification product was digested by XhoI and BglII before being cloned in the sites XhoI-BglII of the pGmAc plasmids carrying the sequence coding for the signal peptide, yielding the plasmids pBCκ.

The composition of the ligation mixture is the following: 1 µg of the plasmid pCmAc cleaved by XhoI and BglII; 200 ng of the Cκ fragment that had been amplified and digested by BglII and XhoI, 2 µl of l0X ligase buffer (BOEHRINGER), distilled water q.s.p. 19 µl, 1 unit (1 µl) of ligase (BOEHRINGER).

Incubation is carried out at 22°C for 2 hours; the ligation product is used for the transformation of competent E. coli bacteria.

Example 2 - Lambda light chain cassette (pBCλ) (Figure 2):

a. Constant region Cλ:

As for the coding sequence of the constant region Cκ, the coding sequence Cλ was obtained by PCR amplification of the complementary DNA of the messenger RNA from human B lymphocytes.

PCR amplification of the region Cλ was performed in the presence of the primer OPP-HuCλ3', complementary of the end 3' of the Cλ regions and supplying the restriction site BglII and the primer OPP-HuCλ5', complementary of the end 5' of the Cλ regions and supplying the restriction site XhoI.

The sequences of the two primers are the following:

* OPP-HuCλ3':

5'-CCT GTC AGA TCT ATG AAC ATT CTG TAG GGG-3'

(site BglII underlined)

* OPP-HuCλ5' :

5'-CCG CCC TCC CTC GAG CTT CAA-3'

(site XhoI underlined)

After cleavage by the enzymes BglII and XhoI, the amplified sequenc Cλ is ins rted between the sites XhoI and BglII of the plasmid pBCκ, which has previously been subjected to deletion of the CK gene by treatment with the enzymes BglII and XhoI and purification of the 7.8 kb plasmid fragment.

The resultant plasmid is referred to as pBCλ.

The insertion of the constant lambda region was verified by sequencing of the plasmid pBCλ in the presence of the two primers OPP-HuCλ3' and OPP-HuCλ5' .

The lambda chain (Cλ) cassette is intended for the cloning of the variable parts of lambda type light chains.

These variable regions are amplified by PCR using, on the one hand, a primer (OPP-HuVλ5') that hybridizes at the level of the framework 1 of the light chains and enables reconstitution of a site SacI and, on the other hand, a primer (OPP-HuVλ3') which is almost complementary to the primer OPP-HuCλ5' and which enables reconstitution of a site XhoI.

The sequences of these primers are as follows:

* OPP-HuVλ5':

5'-CA(GC)TCTGAGCTCAC(GT)CAG-3'

(site SacI underlined)

* OPP-HuVλ3':

5'- TTG AAG CTC CTC GAG GGA GGG CGG GAA-3'

(site XhoI underlined)

Example 3 - Heavy chain γ1 cassette (pBCγ1) (Figure 3):

a - Transfer plasmid

The plasmid pGm16 [BLANC et al., Virology, 192, 651-654 (1993)] derived from a plasmid in which had been cloned th fragment EcoRI-P of the baculovirus AcMNPV containing the gene p10. Almost the entire coding

sequence was deleted and replaced by a sit BglII enabling ins rtion of expression sequences under the control of the p10 promoter.

b - The signal peptide:

The coding sequence of said peptide is the one of the murine VH gene (NEUBERGER M.S., 1983, EMBO J, 2, 1373-1378):

5'-ATG GGA TGG AGC TGT ATC ATC CTC TTC TTG GTA GCA ACA GCT ACA GGT GTC CAC TTC-3'

It was synthesized chemically in the form of complementary strands such that it can be inserted in a site BglII (Figure 3). The pairing and ligation conditions are identical to those employed for the cloning of the coding sequence of the signal peptide used for the light chain.

c- Human constant regions:

- IgG1 (Cγ1)

The cDNA of the coding sequence of the human Cγ1 was amplified by PCR using the following primers:

* HuCγ1BAC:

5'- CAA GGT ACC ACG GTC ACC GTC TCC - 3'

(site KpnI underlined).

This primer corresponds to a consensus sequence of the murine JH regions (ends 3' of the variable regions of murine heavy chains) and comprises a site KpnI.

* HuCγ1FOR:

5' –GAAGATC TCA TTT ACC CGG AGA CAG GGA G-3'

(site BglII underlined)

The sequence was determined from human Cγ1 sequences. The primer is complementary with the end 3' of human Cγ1 and enables reconstitution after amplification of a site BglII downstream of the stop codon.

The matrix employed for amplifying th human Cγ1 region is th same mixture of cDNA that was used for amplification of th coding sequences Cκ and Cλ.

The amplification product was sequenced and cloned in the transfer vector pGm16 carrying the sequence coding for the signal peptide. The resultant construction was referred to as pBCγ1 (Figure 3).

For amplification of the constant regions of the immunoglobulins IgG2, IgG3, IgG4, IgE, IgM and IgA, one uses as the 5' primer, the primer HuCγ1BAC above combined respectively with the following 3' primers:

- IgG2:

HuCγ1FOR,

- IgG3:

HuCγ1FOR,

- IgG4:

HuCγ1FOR,

- IgE:

5'- GAAGATC TCA TTT ACC GGG ATT TAC AGA- 3',

- IgM:

5'-GAAGATCTCA TTT ACC GGT GGA CTT GTC GTC-3',

- IgA:

5'-GAAGATCTCA GTA GCA GGT GCC GTC CAC CTC-3'

For the constant regions of IgG1, 2, 3 and 4, the same primer is used on 3' because of the large amount of conservation of sequences among the different subclasses in this region. For the primers employed for IgE, IgM and IgA, the BglII cleavage sites introduced are underlined.

Example 4 - Expression of a chimeric antibody (mouse-human) in th ins ct cell inf ct d by a v ctor in accordance with th inv ntion:

MAb K20 is a murine antibody produced by a hybridoma. It is directed again the β subunit of the CD29 receptors of the lymphocyt s [BOUMSELL et al., J. Exp. Med. 152, p. 229 (1980)]. A recombinant baculovirus in accordance with the invention was used to express a K20 chimeric antibody having the variable regions of the original K20 and the human constant regions stemming from the cassettes pBC κ and pBC γ1.

1. Cloning of the variable region of the light κ chain of K20:

The total RNA of the hybridoma was extracted using the "RNA Extraction Kit" of PHARMACIA, and an inverse transcription was performed using the primer VKFOR (First-Strand cDNA Synthesis Kit: PHARMACIA):

* VKFOR:

5'- CCG TTT GAT CTC GAG CTT GGT CCC 3'

(site Xho1 underlined)

This primer is complementary to the consensus sequence at the end 3' of the variable region Vκ of the murine genes. It is intended to amplify the Vκ rearranged with the Jκ1 or Jκ2 junctions (which are the most plentiful) but also with those rearranged with the Jκ4 or Jκ5 junctions.

The cDNA was amplified by PCR using, on the one hand, the primer VκFOR and, on the other hand, the primer Vκ2BAC:

* Vκ2HAC:

5'- GAC ATT GAG CTC ACC CAG TCT CCA -3'

(site SacI underlined)

The sequence of this primer is identical to a previously published sequence [WINTER and CLACKSON, Nature, 352, p. 624 (1991)]. Vκ2HAC has the capability of amplifying the murine variable regions Vκ3, Vκ4 and Vκ6.

After amplification of the region VκK20, a SacI-XhoI digestion of the amplification product was p rform d and the resultant fragment was c.loned

between the sites SacI and XhoI of the light chain plasmid pBCκ, yielding the plasmid pBVκK20HuCκ (Figur 4a).

2- Cloning of the variable region of the light chain γ1 of K20:

Inverse transcription on the total RNA extracted from the hybridoma producer of K20 was performed using the primer VHFOR. This primer as well as the primer VH1BAC were then used to amplify the VH from the cDNA:

* VHFOR:

5' - TGA GGA GAC GGT GAC CGT GGTaCC TTG GC-3'

(Site : KpnI underlined)

* VH1BAC:

5'-AG GT(C/G) (A/C)A(A/G) CTG CAG (C/G)AG TC(A/T) GG-3'

(Site PstI underlined)

VHFOR is identical to a primer described by ORLANDI et al. (1989. Proc. Natl. Acad. Sci. USA 86, 3833-3837), with the sole difference located at the level of the "a" which replaces a "C" (cf. site KpnI underlined above).

After amplification and digestion by PstI and KpnI, the VH region of K20 was inserted in the heavy chain plasmid at the level of the sites PstI-KpnI. The charged plasmid was named pBVHK20HUCγI (Figure 4B).

3 - Construction of a recombinant virus producing chimeric K20 (Figure 5)

a - Insertion of the heavy chain:

The charged plasmid pBVHK20HUCγI was used in co-transfection with the DNA from a modified baculovirus called AcSLp10 [CHAABIHI et al., I. Virol., 67, 2664-2671 (1993)], which is lacking in the polyhedrin gene (promoter + coding sequence) but carries the coding sequence of polyhedrin under control of the p10 promoter in the natural locus p10. Since this virus produces polyhedrosis in infected c lls, th r combination at the level of the

p10 locus can thus be asily detect d. The co-transfection conditions are the following: 500 ng of viral DNA are mixed with 5 µg of plasmid DNA and 40 µl of DOTAP solution (BOEHRINGER) in 3 ml of serum-free culture medium for insect cells. This mixture is used to cover 4 x 10^6 Sf9 cells (ATC C35CRL 1711); after 4 hours of contact, the co-transfection mixture is replaced by 4 ml of complete medium and incubation is carried out at 27°C for 5 days.

After the co-transfection, the virus producing the heavy chain of the chimeric antibody K20 under the control of the p10 promoter was purified using the lysis plate technique. This virus was named AcSLpI0-K20H.

b - Insertion of the light chain:

The charged plasmid pBVκK20HUCκ was used in co-transfection with the DNA from the modified baculovirus AcSLp10-K20H.

The recombinant doubles were selected by the limit dilution technique combined with ELISA and/or molecular hybridization.

After the co-transfection, a range of dilutions is made from the infectious supernatant, and each dilution is used for the infection of insect cells. Three days after the infection, the supernatants are tested on ELISA to determine the presence of correctly assembled human-type antibodies. The supernatants from the wells that are the most positive for the highest dilutions are in turn diluted and used to infect other cultures; after several dilution/infection cycles, the supernatants enriched in baculovirus producing the entire antibody are spread on a cell sheet and cloned by the lysis plate meth.od.

Example 5 - Production and purification of the K20 antibody

The double-recombinant virus was amplified bya series of passages over insect cells in culture. The viral stock was then used to infect a culture agitated in a spinner (500 ml of culture at 106 cells per ml).

After 72 hours of infection, the culture was collected and centrifuged at 1000 g in order to clarify the supernatant. The supernatant was concentrated to 1/3 of its initial volume by centrifugation through a membrane having a single 30-kDa slit (CENTRIPEP 30, Amicon) (first centrifugation: 1000 g, 20°C, 30 minutes: elimination of the filtrate; second centrifugation: 1000 g, 20°C, 20 minutes).

The solution was equilibrated in a fixation buffer on protein A, by dilution in this buffer followed by an additional concentration by centrifugation (dilution buffer: glycerol 1.5 M, NaCl 3M; pH 8.9). The equilibrated solution is then passed into a column of protein A, itself equilibrated in the same buffer as the K20 solution. After rinsing of the column, the antibody is eluted by an elution buffer (acetate 0.1 M, NaCl 0.5 M; pH 3). The elution is followed by measuring the OD at 280 nm. The fractions containing the antibodies were mixed and concentrated by centrifugation through a membrane with a single 10-kDa slit (CENTRIPEP 10, AMICON). The concentrated solution is diluted with PBS and then reconcentrated in the same manner. The resultant antibody solution is stored at +4°C in PBS (137 mM NaCl; 2.7 mM KCl; 4.3 mM Na_2HPO_4-$7H_2O$; 1.4 mM KH_2PO_4).

The amount of antibodies was determined byelectrophoresis on polyacrylamide-SDS gel, using as control a commercial human IgG1 (SIGMA). This investigation showed that the unreduced chimeric antibody (disulfide bridges intact) migrates to the same level as the control human antibodies.

After treatment with dithiothreitol (DTT), two bands appeared that corresponded to the heavy and light chains, and migrating to the same level as the chains from the control human antibody that had also been reduced in DTT.

In order to confirm the preceding results, the proteins from the fractions comprising the antibodies were transferred to a nitrocellulose membrane, and

the Hand L chains were d tected by specific antibodies for the human Cγ1 and Cκ regions.

This test demonstrated that the antibodies produced by the insect cells are definitely constituted of Hand L chains and that the constant regions of these chains are recognized by the specific antibodies.

Exarnple 6- Test of the activity and specificity of the K2O produced by the insect cells:

The monoclonal antibody K20 is directed against the β1 subunit of the human integrines (receptor CD29) located at the surface of the lymphocytes. The fixation of K20 on the molecule CD29 inhibits the proliferation of activated human T4 lymphocytes [GROUX et al., Nature, 339, 152-154 (1989)]. The suppressive effect of K20 on T cell proliferation provides a basis for envisaging the use of this antibody in the prevention of graft rejection. In order to test the activity of chimeric K20 antibody produced in accordance with the invention, two types of experiments were carried out: immunofluorescence and inhibition of T lymphocyte proliferation. The experimental protocols employed have essentially been previously described [GROUX et al., Nature 339, 152-154 (1989); TICCHIONI et al., Journal of Immunology 151, 119-127 (1993)].

1 - Immunofluorescence experiments:

Human lymphocytes carrying the CD29 receptor were fixed on glass slides then incubated in the presence of the chimeric K20 produced in accordance with the invention, of K20 of murine origin or of buffer only.

After a series of rinsings, one added either a fluorescent specific secondary antibody of the chimeric K20 produced in accordance with the invention or a fluorescent specific secondary antibody of the K20 of murine origin.

After an additional rinsing, the preparations were examined under the microscope to visualize the fluorescence which showed that the chimeric K20 produced in accordance with the invention becomes attached in a specific manner to the lymphocytes carrying CD29, like the positive control K20 of murine origin; no fluorescence was detected in the absence of the K20 antibodies.

2- Inhibition of the proliferation of $CD4^+$ lymphocytes by K20:

Human CD4+ lymphocytes were activated by an anti-CD3 antibody in the presence of interleukin-2. Chimeric K20 antibodies in accordance with the invention or K20 of murine origin were added (20 µg/ml) to examine their effect on lymphocyte proliferation. A test with a non-inhibitory antibody [GROUX et al., Nature, 339, p. 152 (1989)] was carried out as a negative control.

Proliferation is measured by counting the amount of ^3H-thymidine incorporated after 4 days of culture following the treatments with the antibodies. A level of inhibition of proliferation of 50 to 70% was found when the cells were incubated with the K20 antibody produced in accordance with the invention.

The K20 antibody of murine origin (positive control) also inhibits proliferation of the activated lymphocytes at the same levels: 50.to 70%.

CLAIMS

1) Recombinant baculovirus constituting an expression vector that can be used for the production of immunoglobulins in an insect cell, and characterized in that it comprises:

- an expression cassette comprising a sequence coding for at least one part of an immunoglobulin H chain, which sequence is placed under transcriptional control of a first strong baculovirus promoter or a derivative of said first promoter, and

- an expression cassette comprising a sequence coding for at least one part of an immunoglobulin L chain, which sequence is placed under transcriptional control of a second strong baculovirus promoter or a derivative of said second promoter;

the first and the second promoters are two different promoters or derivatives of different promoters and one of the promoters being located at the site occupied in the wild baculovirus by the polyhedrin promoter and the other promoter is located at the site occupied in the wild baculovirus by the p10 promoter.

2) Recombinant baculovirus according to Claim 1, characterized in that each expression cassette comprises:

(i) a strong baculovirus promoter and, under the control of the said promoter:

(ii) a sequence coding for a signal peptide;

(iii) a sequence coding for a variable immunoglobulin domain;

(iv) a sequence coding for a constant domain of an immunoglobulin H or L chain.

3) Recombinant baculovirus according to Claim 1, characterized in that each expression cassette comprises:

(i) a strong baculovirus promoter and, under the control of the said promoter:

(ii) a sequence coding for a signal peptide;

(iii) at least one site allowing the insertion of a sequence coding for a variable domain of immunoglobulin between the sequence coding for the constant part and the sequence coding for the signal peptide;

(iv) a sequence coding for at least one part of a constant domain of immunoglobulin.

4) Recombinant baculovirus according to any of Claims 2 or 3, characterized in that the sequence coding for a signal peptide placed under the control of the first promoter is different from the sequence coding for a signal peptide placed under the control of the second promoter.

5) Recombinant baculovirus according to any of Claims 2 to 4, characterized in that at least one of the sequence coding for a signal peptide codes for ommunoglobulin peptide signal.

6) Recombinant baculovirus according to any of Claims 2 to 5, characterized in that the sequence coding for the constant immunoglobulin domain is a sequence of human origin.

7) Recombinant baculovirus according to any of Claims 1 to 6, characterized in that one of the promoters is the p10 promoter or one of its derivatives, and the other is the polyhedrin promoter or one of its derivatives.

8) Insect cells characterized in that they are infected by a recombinant baculovirus according to any of Claims 1 to 7.

9) Method for the preparation of an immunoglobulin, characterized in that insect cells infected by a baculovirus according to any of Claims 1, 2 or 4 to 7 are cultured and said immunoglobulin is extracted from the culture medium.

10) Immunoglobulin, characterized in that it can be obtained by the method according to Claim 9.

11) Method for the preparation of a recombinant baculovirus according any of Claims 1 to 7, which method is characterized in that:

- one prepares a first transfer plasmid comprising a sequence coding for at least one part of an immunoglobulin H chain, under transcriptional control of a first strong baculovirus promoter, or a derivative of said first promoter;
- one prepares a second transfer plasmid comprising the sequence coding for at least one part of an immunoglobulin L chain, under transcriptional control of a second strong promoter of the said baculovirus, or a derivative of said second promoter;

the first and second promoters being two different promoters or derivatives of different promoters;

- one carries out the homologous recombination of the two plasmids with baculovirus DNA;
- after replication of the viral DNA in transfected cells, one proceeds to the selection of the recombinant baculoviruses that have integrated the heterologous sequence.

12) Method according to Claim 11, characterized in that ach transfer plasmid us d carries an ins rt comprising an xpression cassette such as defined in any of Claims 1 to 7 and, on both sid s of this cassette, baculovirus sequences homologous with those of the regions flanking the portion of the viral genome which it is the intention to replace by insertion of the said cassette.

13) Method according to Claim 11, characterized in that the said baculovirus sequences are homologous with those of the regions flanking the p10 gene, or homologous with those of the regions flanking the polyhedrin gene.